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**Investigating The Colocalization Of The Follicle Stimulating
Hormone Receptor With Caveolin In Lipid Rafts**

By: David Wood

Advisor: Professor Brian Cohen

Introduction

Located in the brain and stimulated by releasing hormones from the hypothalamus, the anterior pituitary gland secretes hormones into the blood stream that get dispersed throughout the body resulting in a physiological change. One of those hormones is the human follicle-stimulating hormone (FSH), which is a glycoprotein that is essential for reproduction in both males and females (Dias, James). FSH is a dimeric protein made up of two pieces, an alpha subunit, and a beta subunit. The alpha subunit is familiar to all glycoprotein hormones, such as luteinizing hormone, and the thyroid-stimulating hormone. The beta subunit is specific to FSH and is responsible for FSH interaction with its receptor.

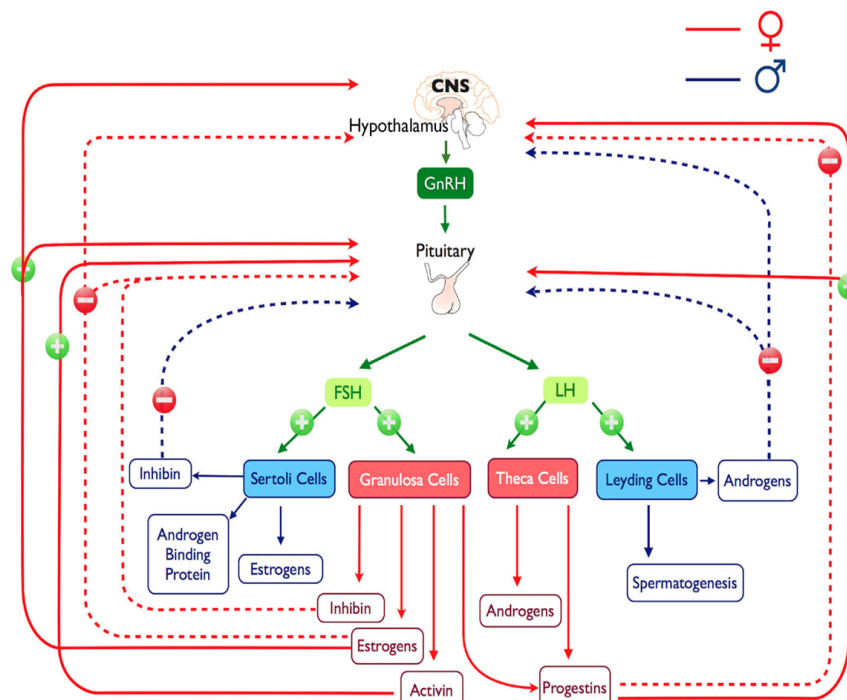


Figure 1. A description of the signaling pathways and feedback loops involved in reproduction specific to FSH (Frontiers)

Mentioned above, the hypothalamus secretes a releasing hormone to stimulate the anterior pituitary to release FSH. The

specific releasing

hormone is the

gonadotrophin-releasing hormone (GnRH). It's secreted in a pulsatile manner, and

the size and frequency of the pulses dictate the level of FSH secretion. In addition to GnRH, FSH is also regulated through negative and positive feedback. When FSH reaches its target cells it not only stimulates reproductive growth, it stimulates production of inhibin, which decreases production of FSH. Inhibin also decreases activin production, a promoter of FSH (Dias, James). Progesterone, estradiol and follistatin are additional inhibitors of FSH providing negative feedback on the FSH pathway. Figure 1 shows the entire positive and negative feedback loop, as well as the targets of FSH and LH.

The cardinal rule for all hormones is that each hormone must have a receptor, and after being secreted by the basophil cells in the anterior pituitary gland, FSH interacts with the follicle-stimulating hormone receptor (FSHR) in its target cells (Dias, James). In males, the target cells are the Sertoli cells, located in the testes, and in females the target cells are granulosa cells in the ovaries. In males, activation of the FSHR leads to Sertoli cell growth, and maximal stimulation of spermatogenesis; the production and maturation of sperm. In females activation of FSHR in granulosa cells is essential for the proliferation, growth, and differentiation of ovarian follicles beyond the antral stage (Yu, F). Granulosa cells surround the oocyte, which is a premature egg. They stimulate its growth in an FSH dependent manner. Once the egg has matured enough, and the granulosa cells have differentiated, they receive signals from the anterior pituitary in the form of luteinizing hormone (LH). This causes the egg to be released from the follicle and the granulosa cells surrounding it. This is considered the point of ovulation.

The FSHR is a 75 kDa MW G protein coupled receptor (GPCR); a transmembrane protein embedded on the cell membrane responsible for signal transduction (Chini, B.). GPCR's are characterized by an extracellular N-terminus or extracellular domain

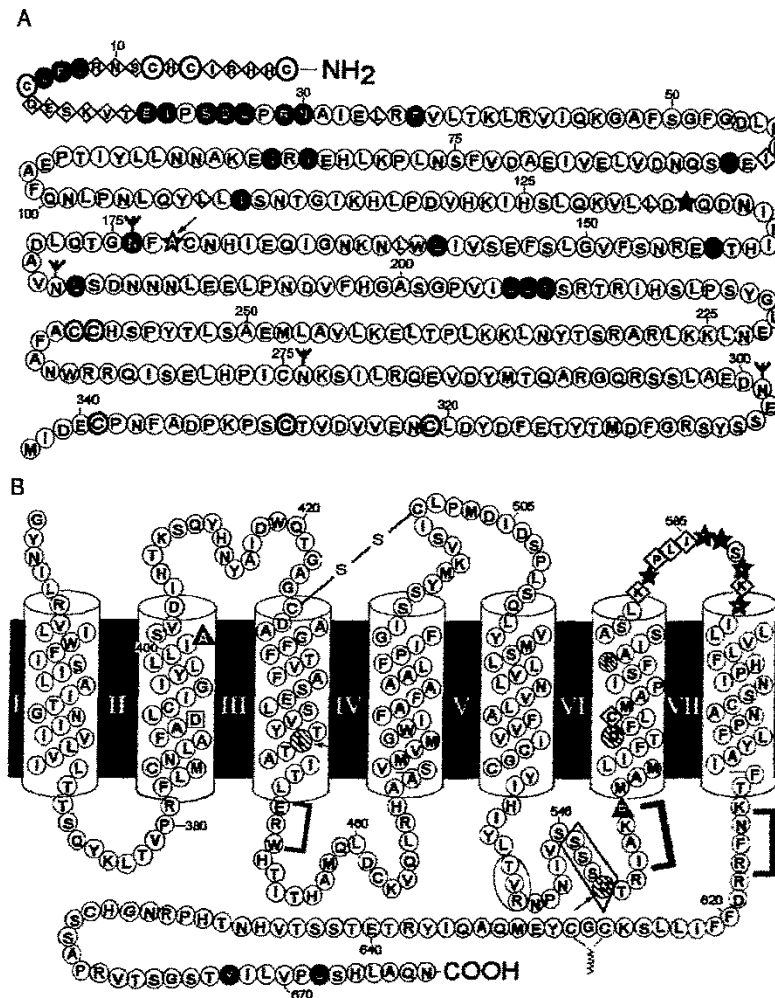


Figure 2. A schematic layout of the FSHR structure in amino acids

affinity and specificity, such that only FSH can activate the receptor. The binding of FSH to the N-terminus of the FSH receptor activates the GPCR and a signal is transduced. Upon activation, the intracellular C-terminus interacts with a heterotrimeric g-protein, which is bound to the cell membrane in close proximity to the FSHR. This heterotrimeric g-protein consists of three subunits; alpha (α), beta

(ECD), connected to a 7-alpha-helical transmembrane spanning domain which has 3 extracellular loops and 3 intracellular loop finally connected to an intracellular C-terminus (Xuliang, Jiang). The N-terminus is responsible for

binding FSH with high

(β), and gamma (γ) and once activated, the subunits dissociate. The α subunit is responsible for activating adenylyl cyclase, which then converts ATP to cAMP. This is the first step in a complex signaling pathway involving many proteins and signals that result in the physiological changes in granulosa and Sertoli cells, discussed above. The $\beta\gamma$ subunit is responsible for activating β -arrestin, an adapter protein that plays a central role in the desensitization and sequestration of GPCR's (Xuliang, Jiang).

β -arrestin plays an important role, because it's responsible for the signals from the receptor being stopped. The β -arrestin protein functions by binding to the GPCR and sterically hindering the g-protein from interacting with the GPCR (Shukla, et al.). With the steric hindrance, no signal can be transduced and the receptor has been desensitized. In addition to desensitization, β -arrestin also facilitates receptor internalization. Clathrin, a protein involved in endocytosis, can be recruited along with several other proteins to the GPCR by β -arrestin (Shukla, et al.). β -arrestin binds to the GPCR, and following recruitment of several other components of clathrin-dependent internalization machinery, the receptor is able to be internalized inside the cell (Shukla, et al.). β -arrestin is also capable of acting as an E3 ubiquitin-ligase adapter, which in turn allows for ubiquitination of the GPCR and consequential degradation of it (Shukla, et al.).

Once thought to be the only signaling pathway stimulated by the FSHR, cAMP is made from ATP when the G-alpha subunit activates adenylyl cyclase (Chedrese,

Pedro). This is a widely known and characterized pathway as it takes place in many different cells, and upon generation, cAMP transduces a variety of signals. It regulates cell growth and differentiation, as well as gene expression within the cell. One of the main activities of cAMP is to activate cAMP-dependent protein kinase, PKA. PKA is an important effector of cAMP because of its ability to phosphorylate a variety of enzymes. cAMP transduces signals to proteins like RAP1, p38MAPK, and P13K without the help of PKA, but PKA is essential for the important phosphorylation of histone H3, and cAMP response element binding protein (CREB) (Chedrese, Pedro). The CREB pathway is responsible for expressing genes including aromatase and inhibin, which is an enzyme in charge of a key step in the production of estrogen and a protein involved in the feedback pathway, respectively (Dias, James).

This complex signaling cascade all comes back to the activation of the FSHR on the cell membrane. Much is known about the signals transduced by the FSHR, but the location of FSHR on the cell membrane is still unknown. The cell membrane is a spherical, 3-dimensional ring that surrounds the cell protecting it from everything outside of the cell. It's composed of lipids and proteins that primarily serve to protect the cell from the outside environment, transduce signals, and allow specific molecules in and out of the cell (Chini, B). Originally thought to be a mostly homogenous mix of these proteins and lipids, the cell membrane actually has structure and order to it. The primary structure of the membrane is a phospholipid bilayer featuring lipids with hydrophilic heads facing outwards, and hydrophobic

tails facing inward; creating a barrier for two aqueous environments. (Chini, B).

Proteins embedded in the membrane help with transport of signals and molecules through the membrane with additional support from lipids surrounding the protein.

Scattered throughout the membrane are planar domains enriched with saturated fats, cholesterol, and sphingolipids, with a high level of structure and order. These complex domains are called lipid rafts. While lipids can be found all over the cell membrane, lipid rafts differentiate themselves because they are much more ordered and tightly packed; serving as a hub for the congregation of signaling molecules, and protein trafficking across the cellular membrane (Quest, Andrew). Lipid rafts are generally characterized by a 5-fold increase in cholesterol and a high concentration of sphingolipids (Pralle, et al.). Sphingolipids are a class of saturated lipids with a backbone of sphingoid bases that have a high interaction with cholesterol due to their structure and saturated hydrocarbon chain. This elevated level of interaction adds structure to lipid raft domains, and helps them function. These rafts are highly associated with many transmembrane proteins involved in signaling, such as GPCR's. Previous to any protein recruitment, lipid rafts contain small proteins that help recruit large signaling protein complexes like GPCR's, and caveolin is one of those proteins. Caveolins are a small scaffolding class of proteins, and is also responsible for helping form lipid rafts, specifically caveolae.

Caveolae are flask-like invaginations of the cell membrane, and are a specific type of lipid raft. Figure 3 shows the organization of caveolae, and caveolin proteins can be seen supporting its

structure. It has been found that much signal transduction happens at lipid raft and caveolae sites, and caveolin proteins are suspected to be highly involved in

this activity (Quest, Andrew). Caveolae's flask-like invagination

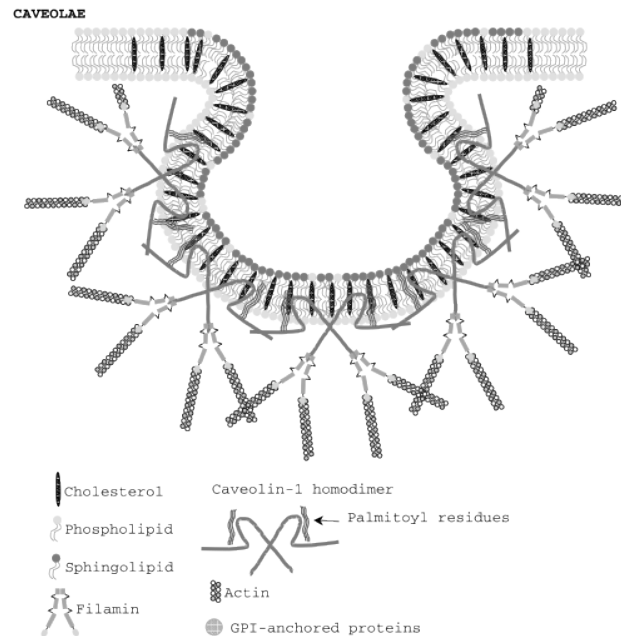


Figure 3. This image shows the design of caveolae lipid rafts and the components that dictate its structure

structure makes for easy internalization. Internalization is one of caveolae's main duties and it has been shown that caveolae are the pathway for internalization of larger molecules (Patel, Hemal). Caveolae are easy subjects to study because they are large enough to be visualized with microscopy, and the caveolin protein is a great marker.

Caveolin is a well-conserved, low-molecular-weight protein with a hydrophobic central domain that possess both lipid and protein binding sites. As said before caveolins are found in caveolae and lipid rafts at very high concentrations. By forming a scaffold on the cell membrane, caveolin allows signaling molecules to

congregate and assemble full signaling complexes (Patel, Hemal). Caveolin proteins are synthesized in the endoplasmic reticulum and form heterooligomers there and in the Golgi before traveling to the plasma membrane (Quest, Andrew). Once reaching the plasma membrane they can fully mature to form higher molecular mass complexes, which then are targeted to the caveolae (Patel, Hemal). The caveolin protein has a hairpin structure, with the N-, and C-terminus interacting with each other and cholesterol in the cell membrane (Okamoto, et al.). This allows them to congregate to form scaffolding structures, and maintain order and structure in lipid raft domains including caveolae. The caveolin protein contains a specific scaffolding motif and in caveolae both that motif, and the process of acylation, regulate the protein composition of the raft and are responsible for protein recruitment (Chedrese, Pedro).

The process of recruiting the signaling molecules to lipid rafts and caveolin is through the caveolin scaffolding domain (Okamoto, et al.), which contains a sequence of amino acids recognized by signaling molecules. These signaling molecules contain a sequence of amino acids called the caveolin-binding motif, and this is the mechanism for most caveolin-mediated sequestration of signaling molecules. Proteins such as GPCR's, receptor tyrosine kinases, and G-protein subunits have this caveolin-binding motif, and are highly involved in signaling (Chini, B.). As mentioned before the caveolin travels from the endoplasmic reticulum and Golgi, to the plasma membrane. This highly specific caveolin binding

motif is what allows the caveolin to target the membrane and caveolae, and is the reason that caveolin is only found in lipid rafts (Chedrese, Pedro).

Conveniently, although the location of FSHR is not known, it is a GPCR and contains a caveolin-binding motif (Xuliang, et al.). Some GPCR's, like the GnRH have already been found to co-localize to lipid rafts and caveolae (Moffett, Serge). Studies show that GnRH can be found in lipid rafts 90% of the time, which gives good reason to suspect that FSHR might do the same (Moffett, Serge). Additionally, the LH receptor, which is a glycoprotein hormone receptor, similar in structure and actions to the FSHR, has also been found to co-localize to lipid rafts (Roess, D.A.). To determine if this is true, we plan to use fluorescently tagged caveolin proteins to be able to see the caveolin using microscopy. To view the FSH receptor under the microscope we will also have to use fluorescence. We will use an antibody to bind to the receptor, and then use a fluorescent antibody that will recognize the primary antibody, giving us fluorescent FSH receptors in our cells. Using high-powered microscopes, the receptor and caveolin will be seen fluorescently, and we will be able to capture images of the receptor and caveolin in the cells. If there is adequate overlap of the two proteins, we can say with confidence that there is co-localization between the two proteins and subsequently a correlation and possible interaction.

Experimental Procedure

Methods and Materials

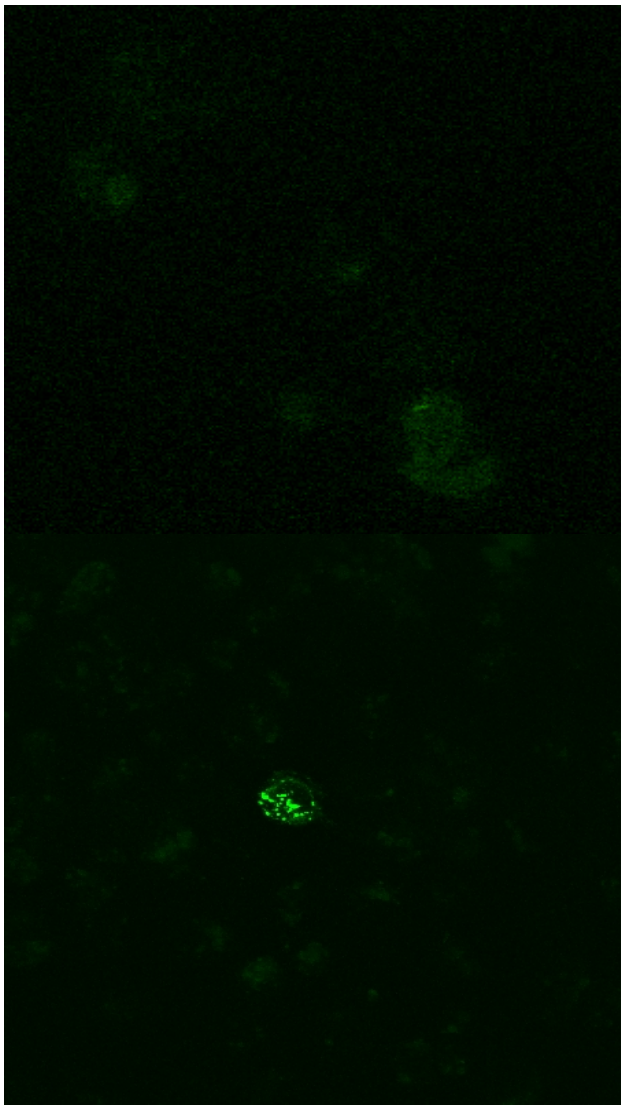
Human Granulosa Cells (HGrC-1^[1]) taken from the lab of Dr. Akira Iwase (Buyasula, et al.) were grown on sterilized coverslips in two 6-well plates. The coverslips were sterilized by soaking in ethanol and flamed. The coverslips were then treated with 0.5 mL (.05 g/mL) poly-D-lysine^[2] and washed 3 times with 2 mL 1x PBS. Following sterilization and coating the HGrC-1 cells were added and grown for 24 hours. The cells were transfected with pYFP caveolin-1 DNA. Wells 1-10 received a 200 μ ^[3]L solution treatment of 6 μ ^[4]L Satisfaction transfection reagent, 12 μ ^[5]L DNA and 178 μ ^[6]L Serum-free media. Two cells were not treated with the GFP-fusion caveolin plasmid, instead serving as controls. After 48 hours of growth, the coverslips were stained for the FSHR.

To fluorescently tag the FSHR we used a goat anti-mouse antibody. The coverslips were washed 3 times with 2 mL of 1x PBS and treated with 1% BSA in 1x PBS for an hour. Following the hour, 250 μ ^[7]L of primary antibody (anti-FSHR mAb 106.105^[8]), taken from the lab of Cheryl Nechamen (Nechamen), was added to the wells in a 1:200 dilution with 1% BSA in 1x PBS. The coverslips were incubated at 4°C for 12 hours and washed again with 2 mL 1x PBS 3 times. The coverslips were then incubated in 250 μ ^[9]L of secondary antibody (goat anti-rabbit Alexa 594) at a 1:500 dilution in 1% BSA in 1x PBS for 1 hour at room temperature. After 3 washes with 2 mL 1x PBS the coverslips were removed from the wells.

A drop of Prolong-Gold Antifade^[10] was added to the microscope slide and the coverslips were placed on the slide with the cell side facing down. Confocal

microscopy was used to visualize the staining. Images were taken with two filters, the first filter allowed us to see receptor fluorescence which appeared red, and the second filter allowed us to see caveolin fluorescence which appeared green. The two images were then transposed, and yellow coloring denoted red and green overlap. The goal was to see red and green overlap, which meant that the caveolin and receptor were localized together in the cell.

Results



Images were taken to identify fluorescence in our transfected HGrC-1 cell line. We looked for two different colors of fluorescence, with green fluorescence identifying and fluorescent caveolin that was transfected into the cell, and red fluorescence being the antibody that identified the FSHR. Image (1) and Image (2) show the difference between a successful transfection (2), and an unsuccessful transfection (1) using a filter for green fluorescence. There is a clear

Figure 4. A comparison of unsuccessfully transfected cells (Top Image 1) with a successfully transfected cell (Bottom Image 2)

contrast between the true green fluorescence and the almost undetectable fluorescence in the un-transfected slide.

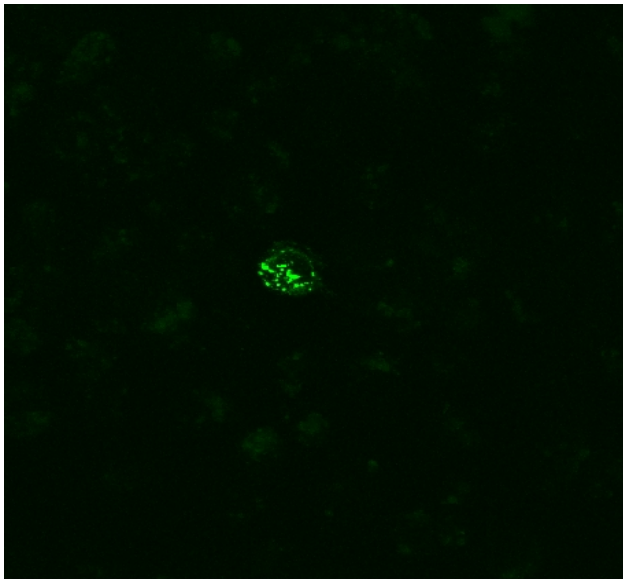
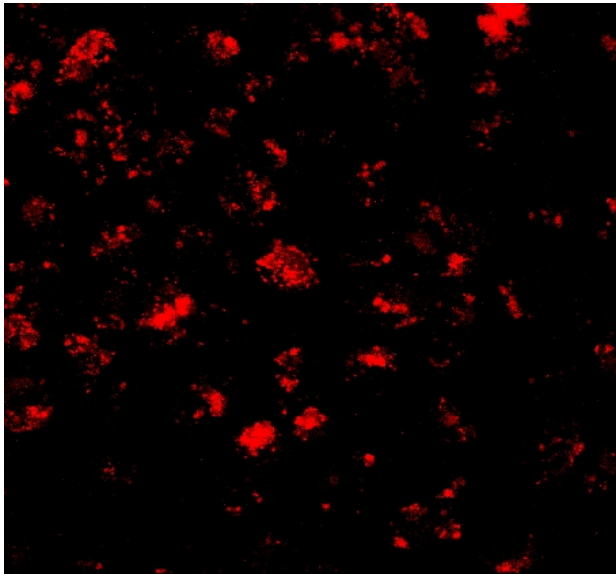


Figure 5. A juxtaposition of two images using two different filters for red receptor fluorescence (Image 3 top), and green caveolin fluorescence (Image 4 Bottom).

Using two filters, one for red fluorescence (FSHR), and the previously mentioned green fluorescence (caveolin), the confocal microscope captured two images with the two separate filters, and then transposed them together. Once put together, yellow coloring would mark the areas where caveolin and the FSHR overlapped. Images 3 and 4 are pictures of the same area on the slide just with different filters for fluorescence.

There is a clear difference in amount of fluorescence between the two,

and the reasons for that will be discussed later in this paper. Image 5 displays the overlapping of caveolin and FSHR with yellow coloring. The bright fluorescence of green caveolin allows caveolin to still be visualized in the transposed image (5), so that there are some areas where caveolin is and the FSHR is not. If there was a

perfect overlap of caveolin and FSHR, there might be a possibility that the red receptor staining is polluting the image (4) of caveolin fluorescence. Instead, there is clearly caveolin in places that FSHR is not, which allows us to say with certainty that the green fluorescence is successfully transfected caveolin.

Due to the difficulty of inserting a plasmid into our HGrC-1 cells (transfecting caveolin), not every cell was successfully transfected with our GFP-fusion caveolin plasmid. Image 3 shows almost every cell in the picture, as the fluorescent antibody for the FSHR bound at a high affinity for FSHR. It tells us that there are clearly un-transfected cells in Image 4, which also verifies the successful transfection of the one fluorescent green cell in Image 4. It is hard to believe that the yellow is an accurate depiction of colocalization because the image is a 2-dimensional picture, but when the confocal microscope takes images in slices of the cell, and then creates a 3-dimensional representation, the yellow appears in specific areas throughout the cell. The 3-dimensional representation tells us that both the FSHR and caveolin are found together in lipid rafts. Image 5 is an example of one slice in the 3-dimensional representation of the transfected cell. There is clear yellow coloring, with

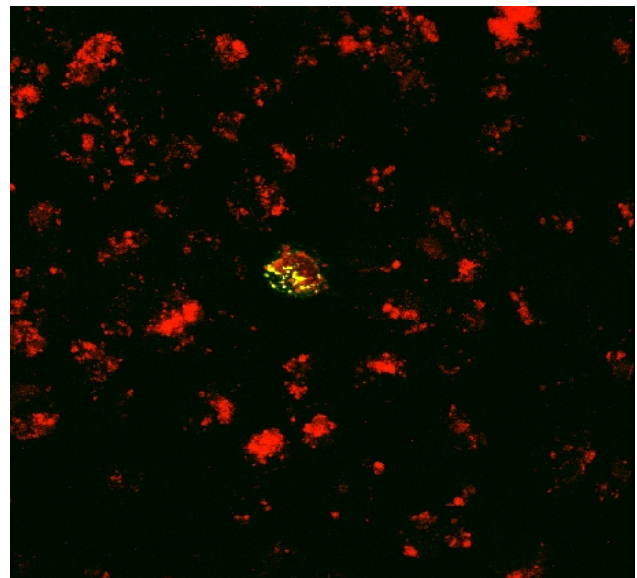


Figure 6. Image 5 is the combination of the two images so that yellow coloring can be visible and evidence of caveolin and FSHR overlap

some yellow spotting around the outside of the cell, outlining the cell membrane.

Discussion

Caveolin and the FSHR localize together in lipid rafts- The goal and hypothesis of my research was that the FSHR and caveolin colocalize in caveolae lipid rafts. Our results, using the selected images from above, provide sufficient evidence that caveolin colocalizes with the FSHR in caveolae lipid rafts.

The yellow coloring is an indication of overlap of caveolin and FSHR. The reason we used fluorescent caveolin to prove that FSHR is found in caveolae lipid rafts is because caveolin is only found in lipid rafts (Patel, Hemal). The overlap indicates that caveolin and the FSHR are in the same area together, and if caveolin is only found lipid rafts, then the area that they're found together in must be lipid rafts.

Image (1) of the results section is a demonstration of an unsuccessfully transfected cell. With a closer look, there is green fluorescence present in some of the cells in Image (1), which originally was thought to be caveolin fluorescence. This however, was proven not to be actual fluorescent caveolin after we found the correctly transfected HGrC-1 cell in Image (2). We can attribute the green fluorescence in Image (1) to being background fluorescence instead, picked up from the confocal microscope. It does however provide good evidence for the brilliant green fluorescence found in Image (2) to be actual fluorescent caveolin. The green fluorescence visible in image (2) is found in spots that are seemingly dense areas of

caveolin in lipid rafts, as well as highlighting the cell membrane all around the edge of the cell.

Evidence that the FSHR is found in lipid rafts opens up many more questions about the mechanics of signal transduction in the FSH pathway. If lipid rafts and caveolae are sites of high signal transduction hypothesizing that lipid rafts are the area of FSHR stimulation would be an accurate, but that's not necessarily the case. Caveolae are rafts that can be involved with internalization^[11], so an alternate hypothesis FSHR's are recruited to caveolae so that the receptor can be internalized and disabled. This could mean that the receptor is stimulated in a separate domain, and brought to caveolae only for internalization. One way to study that would be to stimulate the cell with FSH first, so that the receptor is activated, and then use fluorescence again to see if there is still colocalization of caveolin and the FSHR. If there were, this would disprove the internalization theory^[12].

Another question that could be asked about these results is caveolin's role in the FSHR being present in lipid rafts. The yellow coloring of fluorescence proves that caveolin and the FSHR are found together in lipid rafts, but it doesn't tell us anything about the interaction between them. Among a few other GPCR's, FSHR is one of the only GPCR's to have a caveolin interaction motif. The caveolin interaction motif is a conserved domain on the FSHR's transmembrane domain, and this motif allows FSHR and caveolin to interact with each other. Caveolins action as a recruiter molecule could mean that caveolin actually facilitates the recruitment of FSHR to

caveolae and lipid rafts, and therefore must be present for FSHR to be stimulated. A good test for this interaction can be done in two ways. The first would be a successful mutation of the caveolin interaction motif in the FSHR to see if preventing the interaction prevents successful stimulation of FSHR. This is currently an experiment happening in our lab, to design a mutated FSHR and prevent interaction. Attempting the stimulation of FSHR in caveolin knockout cells would also allow us to see what would happen to the FSHR without caveolin completely^[13]. This has not been studied yet, and is an option for future research. It is although, a less practical technique for outside of lab, because caveolin is crucial for other signal transduction processes, and would most likely result in the death of the cells.

In addition to fluorescence, experiments using coimmunoprecipitation would also be able to test whether or not caveolin and the FSHR interacted. By tagging the receptor with an antibody, and studying the size of the isolated protein with a western blot could tell us if they interacted. If the size of the protein was similar in weight to the FSHR and caveolin combined, we could safely say that they precipitated together. ^[14]Coimmunoprecipitation would be one more step to proving an interaction between caveolin and FSHR. Our lab is currently working on this.

The colocalization of FSHR and caveolin in lipid rafts is a big advance in the field of FSHR research. Finding the site of the receptor on the membrane is progress towards developing additional fertility techniques. The FSHR's role in fertility is one that can be regulated to change the outcomes of reproduction. If we can successfully

block the receptor or promote receptor stimulation with the knowledge we now have, we can establish fertility in those originally infertile, or prevent unwanted pregnancies. Knowing where the receptor lies on the cell membrane could allow us to block signal transduction in lipid rafts, preventing receptor signaling inside of the cell.

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